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(19) (CA) APPLICATION FOR CANADIAN PATENT (12)

(54) Fusion Proteins with Immunoglobulin Portions, the Preparation and Use Thereof

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Description

5 fusion proteins with immunoglobulin portions, the  
preparation and use thereof

10 The invention relates to genetically engineered soluble  
fusion proteins composed of human proteins not belonging  
to the immunoglobulin family, or of parts thereof, and of  
various portions of the constant region of immunoglobulin  
molecules. The functional properties of the two fusion  
partners are, surprisingly, retained in the fusion  
protein.

15 EP-A 0 325 262 and EP-A 0 314 317 disclose corresponding  
fusion proteins composed of various domains of the CD4  
membrane protein of human T cells and of human IgG1  
portions. Some of these fusion proteins bind with the  
same affinity to the glycoprotein gp120 of human immuno-  
20 deficiency virus as the cell-bound CD4 molecule. The CD4  
molecule belongs to the immunoglobulin family and,  
consequently, has a very similar tertiary structure to  
that of immunoglobulin molecules. This also applies to  
the  $\alpha$  chain of the T-cell antigen receptor, for which  
such fusions have also been described (Gascoigne et al.,  
25 Proc. Natl. Acad. Sci. USA, vol. 84 (1987), 2937-2940).  
Hence, on the basis of the very similar domain structure,  
in this case retention of the biological activity of the  
two fusion partners in the fusion protein was to be  
expected.

30 The human proteins which are, according to the invention,  
preferably coupled to the amino terminus of the constant  
region of immunoglobulin do not belong to the immuno-  
globulin family and are to be assigned to the following  
classes: (1) membrane-bound proteins whose extracellular  
35 domain is wholly or partly incorporated in the fusion.  
These are, in particular, thromboplastin and cytokine





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arginine residue. Sequences which contain the described tetrapeptides were introduced first by Nagai and Thøgersen in a hybrid protein by genetic engineering means (Nagai, K. and Thøgersen, H.C., Nature, vol. 309 (1984), 810-812). These authors were able to show that the proteins expressed in *E. coli* actually are specifically cleaved by factor Xa. However, there is as yet no published example of the possibility of such proteins also being expressed in eukaryotic and, especially, in animal cells and, after their purification, being cleaved by factor Xa. However, expression of the proteins according to the invention in animal cells is preferable because only in a cell system of this type is there expected to be secretion of, for example, normally membrane-bound receptors as fusion partners with retention of their natural structure and thus of their biological activity. Secretion into the cell culture supernatant facilitates the subsequent straightforward purification of the fusion protein.

The invention thus relates to genetically engineered soluble fusion proteins composed of human proteins not belonging to the immunoglobulin family, or of parts thereof, and of various portions of the constant regions of heavy or light chains of immunoglobulins of various subclasses (IgG, IgM, IgA, IgE). Preferred as immunoglobulin is the constant part of the heavy chain of human IgG, particularly preferably of human IgG1, where fusion takes place at the hinge region. In a particular embodiment, the Fc part can be removed in a simple way by a cleavage sequence which is also incorporated and can be cleaved with factor Xa.

furthermore, the invention relates to processes for the preparation of these fusion proteins by genetic engineering, and to the use thereof for diagnosis and therapy.



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The invention will now be described in relation to the drawings, in which:

Figure 1 shows two oligonucleotide probe molecules  
 5 used in cloning of thromboplastin cDNA;

Figure 2 shows the nucleotide sequence of clone 2b-  
 Apr5 with the thromboplastin amino acid sequence deduced  
 therefrom;

Figure 3 shows two oligonucleotide sequences which  
 10 are partially homologous with the sequence of the coding  
 strand (A), and with the non-coding strand (B) of  
 thromboplastin cDNA;

Figure 4 shows the restriction map of plasmid  
 pTF1Fc;

Figure 5 shows two oligonucleotide sequences which  
 15 are partially homologous with the sequence of the coding  
 strand (A), and with the non-coding strand (B) of the IL-4  
 receptor cDNA cloned in the vector pOC302/T22-8;

Figure 6 shows the restriction map of plasmid  
 20 pIL4Rfc;

Figure 7 shows two oligonucleotide sequences A and  
 B which are partially homologous with the sequence of the  
 coding strand (A), and with the non-coding strand (B) of the  
 EPO cDNA cloned in the vector pCX8; and

Figure 8 shows the restriction map of plasmid  
 25 pEPOFc.

Finally, the invention is explained in further examples.



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#### Example 1: Thromboplastin fusion proteins

Blood coagulation is a process of central importance in the human body. There is appropriately delicate regulation of the coagulation cascade, in which a large number of cellular factors and plasma proteins cooperate. These proteins (and their cofactors) in their entirety are called coagulation factors. The final products of the coagulation cascade are thrombin, which induces the aggregation of blood platelets, and fibrin which stabilizes the platelet thrombus. Thrombin catalyzes the formation of fibrin from fibrinogen and itself is formed by limited proteolysis of prothrombin. Activated factor X (factor Xa) is responsible for this step and, in the presence of factor Va and calcium ions, binds to platelet membranes and cleaves prothrombin.

Two ways exist for factor X to be activated, the extrinsic and the intrinsic pathway. In the intrinsic pathway a series of factors is activated by proteolysis in order for each of them to form active proteases. In the extrinsic pathway, there is increased synthesis of thromboplastin (tissue factor) by damaged cells, and it activates factor X, together with factor VIIa and calcium ions. It was formerly assumed that the activity of thromboplastin is confined to this reaction. However, the thromboplastin/VIIa complex also intervenes to activate the intrinsic pathway at the level of factor IX. Thus, a thromboplastin/VIIa complex is one of the most important physiological activators of blood coagulation.

It is therefore conceivable that thromboplastin, apart from its use as diagnostic aid (see below), can also be employed as constituent of therapeutic agents for treating inborn or acquired blood coagulation deficiencies. Examples of this are chronic hemophilias caused by a deficiency of factors VIII, IX or XI or else acute disturbances of blood coagulation as a consequence of, for example, liver or kidney disease. Use of such a



therapeutic agent after surgical intervention would also be conceivable.

Thromboplastin is required as additive to plasma samples in diagnostic tests of coagulation. The coagulation status of the tested person can be found by the one-stage prothrombin clotting time determination (for example Quick's test). The thromboplastin required for diagnostic tests is currently obtained from human tissue, and the preparation process is difficult to standardize, the yield is low and considerable amounts of human starting material (placentae) must be supplied. On the other hand, it is to be expected that preparation of native, membrane-bound thromboplastin by genetic engineering will also be difficult owing to complex purification processes. These difficulties can be avoided by the fusion according to the invention to immunoglobulin portions.





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The thromboplastin fusion proteins according to the invention are secreted by mammalian cells (for example CHO, BHK, COS cells) into the culture medium, purified by affinity chromatography on protein A-Sepharose and have surprisingly high activity in the one-stage prothrombin clotting time determination.

#### Cloning of thromboplastin cDNA

The sequence published by Scarpati et al., Biochemistry, vol. 26 (1987), 5234-5238, was used for cloning the thromboplastin cDNA. Two oligonucleotide probe molecules (see Fig. 1) were derived from this. These two probe molecules were used to screen a cDNA bank from human placenta (Grundmann et al., Proc. Natl. Acad. Sci. USA, vol. 83 (1986), 8024-8028).

cDNA clones of various lengths were obtained. One clone, 2b-Apr5, which is used for the subsequent procedure, codes for the same amino-acid sequence as the cDNA described in Scarpati et al. Fig. 2 depicts the total sequence of the clone 2b-Apr5 with the thromboplastin amino-acid sequence deduced therefrom.

#### Construction of a hybrid plasmid pTF1K coding for thromboplastin fusion protein.

The plasmid pCD48 gamma 1 (BP 0 325 262 A2; deposited at the ATCC under the number No. 67610) is used for expression of a fusion protein composed of human CD4 receptor and human IgG1. The DNA sequence coding for the extracellular domain of CD4 is deleted from this plasmid using the restriction enzymes HindIII and BamHI. Only partial cleavage must be carried out with the enzyme HindIII in this case, in order to cut at only one of the two HindIII sites contained in pCD48 gamma 1 (position 2198). The result is an opened vector in which a eukaryotic transcription regulation sequence (promoter) is followed by the open HindIII site. The open BamHI site is



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located at the start of the coding region for a pentapeptide linker, followed by the hinge and the CH2 and CH3 domains of human IgG1. The reading frame in the BamHI recognition sequence GGATCC is such that GAT is translated as aspartic acid. DNA amplification with thermostable DNA polymerase makes it possible to modify a given sequence in such a way that any desired sequences are attached at one or both ends. Two oligonucleotides able to hybridize with sequences in the 5'-untranslated region (A: 5' GATCGATTACGCTTCGGAACCCGCTCGATCTCGCCGCC 3') or coding region (B: 5' GCATATCTGGATCCCCGTAGAATATTCTCTGAATCCCC 3') of thromboplastin cDNA were synthesized. Of these, oligonucleotide A is partially homologous with the sequence of the coding strand, and oligonucleotide B is partially homologous with the non-coding strand; cf. Fig. 3.

Thus, amplification results in a DNA fragment (827 bp) which contains (based on the coding strand) at the 5' end before the start of the coding sequence a HindIII site, and at the 3' end after the codon for the first three amino-acid residues of the transmembrane region a BamHI site. The reading frame in the BamHI cleavage site is such that ligation with the BamHI site in pCD43 gamma 1 results in a gene fusion with a reading frame continuous from the initiation codon of the thromboplastin cDNA to the stop codon of the heavy chain of IgG1. The desired fragment was obtained and, after treatment with HindIII and BamHI, ligated into the vector pCD4E gamma 1, as described above, which had been cut with HindIII (partially) and BamHI. The resulting plasmid was called pTF1Fc (Fig. 4).

#### Transfection of pTF1Fc into mammalian cells

The fusion protein encoded by the plasmid pTF1Fc is called pTF1Fc hereinafter. pTF1Fc was transiently expressed in COS cells. For this purpose, COS cells were



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transfected with pTF1Fc with the aid of DEAE-dextran (EP A 0 325 262). Indirect immunofluorescence investigations revealed that the proportion of transfected cells was about 25 %. 24 h after transfection, the cells were transferred into serum-free medium. This cell supernatant was harvested after a further three days.

#### Purification of pTF1Fc fusion protein from cell culture supernatants

170 ml of supernatant from transiently transfected COS cells were collected overnight in a batch process in a column containing 0.8 ml of protein A-Sepharose at 4°C, washed with 10 volumes of washing buffer (50 mM tris buffer pH 8.6, 150 mM NaCl) and eluted in 0.5 ml fractions with eluting buffer (93:7 100 mM citric acid; 100 mM sodium citrate). The first 9 fractions were immediately neutralized with 0.1 ml of 2M tris buffer pH 8.6 in each case and then combined, and the resulting protein was transferred by three concentration/dilution cycles in an Amicon microconcentrator (Centricon 30) into TNE buffer (50 mM tris buffer pH 7.4, 50 mM NaCl, 1 mM EDTA). The pTF1Fc obtained in this way is pure by SDS-PAGE electrophoresis (U.K. Lämmli, Nature 227 (1970) 680-685). In the absence of reducing agents it behaves in the SDS-PAGE like a dimer (about 165 kDa).

#### Biological activity of purified TF1Fc in the prothrombin clotting time determination

TF1Fc fusion protein is active in low concentrations (> 50 ng/ml) in the one-stage prothrombin clotting time determination (Vinazzer, H. Gerinnungsphysiologie und Methoden im Blutgerinnungslabor (1979), Fisher Verlag Stuttgart). The clotting times achieved are comparable with the clotting times obtained with thromboplastin isolated from human placenta.



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**Example 2: Interleukin-4 receptor fusion proteins**

Interleukin-4 (IL-4) is synthesized by T cells and was originally called B-cell growth factor because it is able to stimulate B-cell proliferation. It exerts a large number of effects on these cells. One in particular is the stimulation of synthesis of molecules of immunoglobulin subclasses IgG1 and IgG2 in activated B cells (Coffmann et al., Immunol. Rev., vol. 102 (1988) 5). In addition, IL-4 also regulates the proliferation and differentiation of T cells and other hemopoietic cells. It thus contributes to the regulation of allergic and other immunological reactions. IL-4 binds with high affinity to a specific receptor. The cDNA which codes for the human IL-4 receptor has been isolated (Idzerda et al., J. Exp. Med., vol. 171 (1990) 861-873). It is evident from analysis of the amino-acid sequence deduced from the cDNA sequence that the IL-4 receptor is composed of a total of 825 amino acids, with the 25 N-terminal amino acids acting as signal peptide. Mature human IL-4 receptor is composed of 800 amino acids and, like thromboplastin, has a three-domain structure: i) amino-terminal extracellular domain (207 amino acids); ii) transmembrane region (24 amino acids) and iii) cytoplasmic domain (569 amino acids). In the extracellular domain there are six potential sites for N-glycosylation (Asn-X-Thr/Ser). IL-4 receptor has homologies with human IL-6 receptor, with the  $\beta$ -subunit of human IL-2 receptor, with mouse erythropoietin receptor and with rat prolactin receptor (Idzerda et al., loc. cit.). Thus, like thromboplastin, it is not a member of the immunoglobulin family but is assigned together with the homologous proteins mentioned to the new family of hematopoietin receptors. Members of this family have four cysteine residues and a conserved sequence (Trp-Sar-X-Trp-Ser) in the extracellular domain located near the transmembrane region in common.

On the basis of the described function of the IL-4/IL-4



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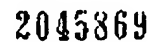
receptor system, there is a possible therapeutic use of a recombinant form of the IL-4 receptor for suppressing IL-4-mediated immune reactions (for example transplant rejection reaction, autoimmune diseases, allergic reactions).

The amount of substance required for therapy makes it necessary to prepare such molecules by genetic engineering. Because of the straightforward purification by affinity chromatography and improved pharmacokinetic properties, according to the invention the synthesis of soluble forms of the IL-4 receptor as immunoglobulin fusion protein is particularly advantageous.

The IL-4 receptor fusion proteins are secreted by mammalian cells (for example CHO, BHK, COS cells) into the culture medium, purified by affinity chromatography on protein A-Sepharose and have, surprisingly, identical functional properties to the extracellular domain of the intact membrane-bound IL-4 receptor molecule.

Construction of a hybrid plasmid pIL-4BFC coding for IL-4 receptor fusion protein.

Cutting of the plasmid pCD4E gamma1 with XhoI and BamHI results in an opened vector in which the open XhoI site is located downstream from the promoter sequence. The open BamHI site is located at the start of the coding regions for a pentapeptide linker, followed by the hinge and the CH2 and CH3 domains of human IgG1. The reading frame in the BamHI recognition sequence GGATCC is such that GAT is translated as aspartic acid. DNA amplification with thermostable DNA polymerase makes it possible to modify a given sequence in such a way that any desired sequences can be attached at one or both ends. Two oligonucleotides able to hybridize with sequences in the 5'-untranslated region (A: 5' GATCCAGTACTCGAGAGAGAGCCGGGCGTGGTGGCTCATGC 3') or coding region



(B: 5' CTATGACATGGATCGCTGCTCGAGGCTCCCTGTAGGACTGTG 3')  
of the IL-4 receptor cDNA which is cloned in the vector  
pDC302/T22-8 (Idzerda et al., loc. cit.) were  
synthesized. Of these, oligonucleotide A is partially  
homologous with the sequence of the coding strand, and  
oligonucleotide B is partially homologous with the non-  
coding strand; cf. Fig. 5. Amplification using thermo-  
stable DNA polymerase results in a DNA fragment (836 bp)  
which, based on the coding strand, contains at the 5' end  
and before the start of the coding sequence an XhoI site, and  
at the 3' end and before the last codon of the extracellular  
domain a BamHI site. The reading frame in the BamHI  
cleavage site is such that ligation with the BamHI site  
in pCD4E gamma 1 results in a gene fusion with a reading  
frame continuous from the initiation codon of the IL-4  
receptor cDNA to the stop codon of the heavy chain of  
IgG1. The desired fragment was obtained and, after  
treatment with XhoI and BamHI, ligated into the vector  
pCD4E gamma 1, described above, which had been cut with  
XhoI/BamHI. The resulting plasmid was called pIL4Rfc  
(Fig. 6).

The fusion protein encoded by the plasmid pIL4Rfc is called pIL4Rfc hereinafter. pIL4Rfc was transiently expressed in COS cells. For this purpose, COS cells were transfected with pIL4Rfc with the aid of DEAE-dextran (EP A 0 325 262). Indirect immunofluorescence investigations revealed that the proportion of transfected cells was about 25 %. 24 h after transfection, the cells were transferred into serum-free medium. This cell supernatant was harvested after a further three days.

500 ml of supernatant from transiently transfected COS



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cells were collected overnight in a batch process in a column containing 1.6 ml of protein A-Sepharose at 4°C, washed with 10 volumes of washing buffer (50 mM tris buffer pH 8.6, 150 mM NaCl) and eluted in 0.5 ml fractions with eluting buffer (93:7 100 mM citric acid: 100 mM sodium citrate). The first 9 fractions were immediately neutralized with 0.1 ml of 2M tris buffer pH 8.6 in each case and then combined, and the resulting protein was transferred by three concentration/dilution cycles in an Amicon microconcentrator (Centricon 30) into TNE buffer (50 mM tris buffer pH 7.4, 50 mM NaCl, 1 mM EDTA). The IL4R<sub>FC</sub> obtained in this way is pure by SDS-PAGE electrophoresis (U.K. Lämmlé, Nature 227 (1970) 680-685). In the absence of reducing agents it behaves in the SDS-PAGE like a dimer (about 150 kDa).

#### Biological activity of purified IL4R<sub>FC</sub>

IL4R<sub>FC</sub> proteins binds <sup>125</sup>I-radiolabeled IL-4 with the same affinity ( $K_d=0.5$  nM) as membrane-bound intact IL-4 receptor. It inhibits the proliferation of IL-4-dependent cell line CTLLHuIL-4RI clone D (Idzarda et al., loc. cit.) in concentrations of 10-1000 ng/ml. In addition, it is outstandingly suitable for developing IL-4 binding assays because it can be bound via its Fc part to microtiter plates previously coated with, for example, rabbit anti-human IgG, and in this form likewise binds its ligands with high affinity.

#### Example 3: Erythropoietin fusion proteins

Mature erythropoietin (EPO) is a glycoprotein which is composed of 166 amino acids and is essential for the development of erythrocytes. It stimulates the maturation and the terminal differentiation of erythroid precursor cells. The cDNA for human EPO has been cloned (EP-A-0 267 678) and codes for the 166 amino acids of mature EPO and a signal peptide of 22 amino acids which is essential for secretion. The cDNA can be used to



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prepare recombinant functional EPO in genetically manipulated mammalian cells and the EPO can be employed clinically for the therapy of anemic manifestations of various etiologies (for example associated with acute renal failure).

Because of the straightforward purification and the improved pharmacokinetic properties, according to the invention synthesis of EPO as immunoglobulin fusion protein is particularly advantageous.

- 10 Construction of a hybrid plasmid pEPOFc coding for erythropoietin fusion protein.

This construction was carried out in analogy to that described in Example 2 (section: "Construction of a hybrid plasmid pIL-4Rfc coding for IL-4 receptor fusion protein"). Two oligonucleotides able to hybridize with sequences in the vicinity of the initiation codon  
 15 (A: 5'-GATCGATCTCGAGATCGGGGTGCACGAATGTCCTGCCCTGGCTGTGG 3') and of the stop codon  
 (B: 5'-CTGGAATCGGATCCCTGTCTCTGCAGGCTCCCTGTGTACAGC 3')  
 20 of the EPO cDNA cloned in the vector pCBS (EP-A 0 267 678) were synthesized. Of these, oligonucleotide A is partially homologous with the sequence of the coding strand, and oligonucleotide B is partially homologous with the non-coding strand; cf. Fig. 7. Amplification  
 25 with thermostable DNA polymerase results in a DNA fragment (598 bp) which, based on the coding strand, contains at the 5' end in front of the initiation codon an XhoI site and in which at the 3' end the codon for the penultimate C-terminal amino acid residue of the EPO  
 30 (Asp) is present in a BamHI recognition sequence. The reading frame in the BamHI cleavage site is such that ligation with the BamHI site in pCD4E gamma 1 results in a gene fusion with a reading frame continuous from the initiation codon of EPO cDNA to the stop codon of the  
 35 heavy chain of IgG1. The desired fragment was obtained and, after treatment with XhoI and BamHI, ligated into





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the vector pCD4E gamma 1, described above, which had been cut with XhoI/BamHI. The resulting plasmid was called pEPORc (Fig. 8).



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NOE 90/B 025

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE  
PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A soluble fusion protein composed of human proteins  
not belonging to the immunoglobulin family, or of  
parts thereof, and of various portions of immuno-  
globulin molecules of all subclasses.
2. A fusion protein as claimed in claim 1, wherein the  
immunoglobulin portion is the constant part of the  
heavy chain of human IgG.
3. A fusion protein as claimed in claim 2, wherein the  
immunoglobulin portion is the constant part of the  
heavy chain of human IgG1 or a protein A-binding  
fragment thereof.
4. A fusion protein as claimed in claim 2 or claim 3,  
wherein the fusion takes place at the hinge region.
5. A fusion protein as claimed in claims 1 - 4, wherein  
the protein fused to immunoglobulin is the extra-  
cellular portion of a membrane protein or parts  
thereof.
6. A fusion protein as claimed in claims 1 - 4, wherein  
the protein fused to immunoglobulin is the extra-  
cellular portion of thromboplastin or parts thereof.
7. A fusion protein as claimed in claims 1 - 4, wherein  
the protein fused to immunoglobulin is the extra-  
cellular portion of a cytokine receptor or growth  
factor receptor or parts thereof.
8. A fusion protein as claimed in claim 7, wherein the  
protein fused to immunoglobulin is the extracellular  
portion of IL-4 receptor or parts thereof.
9. A fusion protein as claimed in claim 7, wherein the  
protein fused to immunoglobulin is the extracellular  
portion of IL-7 receptor or parts thereof.



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10. A fusion protein as claimed in claim 7, wherein the protein fused to immunoglobulin is the extracellular portion of tumor necrosis factor receptor or parts thereof.
- 5 11. A fusion protein as claimed in claim 7, wherein the protein fused to immunoglobulin is the extracellular portion of G-CSF receptor or parts thereof.
12. A fusion protein as claimed in claim 7, wherein the protein fused to immunoglobulin is the extracellular portion of GM-CSF receptor or parts thereof.
- 10 13. A fusion protein as claimed in claim 7, wherein the protein fused to immunoglobulin is the extracellular portion of erythropoietin receptor or parts thereof.
14. A fusion protein as claimed in claims 1 - 4, wherein the protein fused to immunoglobulin is a non-membrane-bound soluble protein or part thereof.
- 15 15. A fusion protein as claimed in claim 14, wherein the protein fused to immunoglobulin is a cytokine or growth factor or part thereof.
- 20 16. A fusion protein as claimed in claim 15, wherein the protein fused to immunoglobulin is erythropoietin or part thereof.
17. A fusion protein as claimed in claim 15, wherein the protein fused to immunoglobulin is GM-CSF or G-CSF or part thereof.
- 25 18. A fusion protein as claimed in claim 15, wherein the protein fused to immunoglobulin is interleukin IL-1 to IL-8 or part thereof.



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19. A fusion protein as claimed in any of preceding claims 1-18, wherein a factor Xa cleavage site is additionally inserted between the immunoglobulin part and the non-immunoglobulin part.
- 5 20. A process for preparing fusion proteins as claimed in any of claims 1 - 19, which comprises introducing the DNA coding for these constructs into a mammalian cell expression system and, after expression, purifying the produced fusion protein by affinity chromatography via the immunoglobulin portion.
- 10 21. The use of the fusion proteins as claimed in any of claims 1 - 19 for diagnosis.
22. The use of the fusion proteins as claimed in any of claims 1 - 19 for therapy.



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23. The fusion protein as claimed in claim 1 and substantially as described herein.



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HQB 9045869

**Abstract of the disclosure**

**Fusion proteins with immunoglobulin portions, the preparation and use thereof**

The invention relates to genetically engineered soluble fusion proteins composed of human proteins not belonging to the immunoglobulin family, or of parts thereof, and of various portions of the constant region of immunoglobulin molecules. The functional properties of the two fusion partners are surprisingly retained in the fusion protein.